FUNCTIONAL ACTIVATION BY GLUCAGON OF GLUCOSE 6-PHOSPHATASE AND GLUCONEOGENESIS IN THE PERFUSED LIVER OF THE FETAL GUINEA PIG

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Received 27 May 1980

1. Introduction

The fetal livers of several species have the enzymic capacity for gluconeogenesis [1–4]. Despite this, de novo glucose synthesis has not been demonstrated, and glucose output from the perfused fetal liver [5–7] is probably the result of glycogenolysis rather than gluconeogenesis. Studies with the perfused fetal guinea pig liver have indicated that at 50 days gestational age no gluconeogenesis occurs in the presence of a range of gluconeogenic substrates, even though the liver has moderate activities of the gluconeogenic enzymes. The reasons for this are unclear. Glucagon is a potent activator of gluconeogenesis in adult livers [8] and stimulated the incorporation of [^{14}C]alanine into glucose by the perfused liver of the near-term (63 day) guinea pig fetus [9]. Thus the effects of glucagon on the perfused fetal guinea pig liver at 50 days have been investigated. The results indicate that glucagon stimulates gluconeogenesis and causes functional activation of glucose 6-phosphatase.

2. Materials and methods

2.1. *Chemicals*

Glucagon was obtained from Novo Industries, Copenhagen. Heparin was from Evans Medical, Speke, Liverpool, and pentobarbital from May and Baker, Dagenham, Essex. NADH, NADPH, NADP$^+$ and all enzymes except amyloglucosidase were obtained from Boehringer (London), Lewes, Sussex. Amyloglucosidase, ATP, ADP, AMP, ITP, IDP, glucose 6-phosphate, phosphoenolpyruvate, dihydroxyacetone phosphate, acetyl phosphate, coenzyme A, oxaloacetate, malonate, glycylglycine imidazole, mecaptoethanol and albumin were obtained from Sigma (London), Kingston-upon-Thames, Surrey. [U-^{14}C]Glucose, [2-^{3}H]glucose and $NaH^{14}CO_3$ were obtained from the Radiochemical Centre, Amersham, Bucks. All other chemicals were from BDH, Poole, Dorset.

2.2. *Determination of enzyme activities*

The methods used have been described for pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose 1,6-diphosphatase and glucose 6-phosphatase [3], hexokinase [10], phosphofructokinase, triose phosphate isomerase [11], pyruvate kinase [12] and glucose 6-phosphate dehydrogenase [13].

2.3. *Liver perfusion*

The livers of 49–51 day fetal guinea pigs were perfused through the umbilical vein in a non-recirculatory system with Krebs bicarbonate buffer as in [14]. Perfusate samples were collected and acidified with $HClO_4$; aliquots were then neutralised with KOH and assayed for glucose enzymatically; ^3H_2O was determined as below. At the end of the perfusion period the livers were removed and freeze-clamped [15]. The frozen tissue (2.5–3 g) was powdered under liquid nitrogen with a ceramic pestle and mortar and deproteinized by thawing into 1 ml 30% (w/v) $HClO_4$ together with 7 ml of H_2O. After removal of the protein precipitate the extract was adjusted to pH 3–4 with 20% (w/v) KOH. Metabolites were assayed by standard methods [16]. Glycogen was determined as the amount of glucose released following incubation with amyloglucosidase at pH 4.4.

2.4. *Determination of ^3H_2O and specific activities*

Aliquots (1 ml) of acidified perfusate samples were

neutralised with KOH and passed through four 0.5 × 2.5 cm columns in tandem consisting of one Dowex 50 (100–200 mesh) H^+ form, one Dowex 1 (50–100 mesh) OH^- form, and two Dowex 1 (50–100 mesh) borate form. Labelled solutes were absorbed by these columns, and ^3H_2O was eluted with H_2O. Aliquots of the eluate were counted for ^3H radioactivity. ^3H specific activity in the 2′-position of glucose 6-phosphate was determined by incubation of 1 ml aliquots of liver extract with 0.2 ml 0.05 M Tris–HCl (pH 8.0) and 1.4 units of phosphoglucose isomerase for 3 h at room temperature, following which the ^3H_2O produced was determined as above. ^{14}C specific activity was determined (for the 1′-position of glucose 6-phosphate) by incubation of 1 ml liver extract with 1 ml 0.05 M triethanolamine–HCl (pH 7.5), 0.050 ml 20 mg $NADP^+$/ml, 0.1 ml 0.6 M Na HCO_3 1.2 units glucose 6-phosphate dehydrogenase and 0.1 units 6-phosphogluconate dehydrogenase. The incubation was carried out in a sealed vessel with a centre well containing 0.2 ml 5 M NaOH for 3 h at room temp.; 0.2 ml 5 M H_2SO_4 was then added to the mixture which was left for a further 3 h. $^{14}CO_2$ released was trapped in the NaOH, which was washed into 1 M barium acetate; the barium carbonate precipitate was resuspended in H_2O and counted for ^{14}C radioactivity. The value for blanks containing 6-phosphogluconate dehydrogenase but no glucose 6-phosphate dehydrogenase was subtracted. The total ^{14}C specific activity was estimated as the ^{14}C dpm measured as described multiplied by 6, per nmol glucose 6-phosphate. In this assay fructose 6-phosphate influenced measured glucose 6-phosphate specific activity by <10%. For each experiment the ^{14}C and ^3H specific activities were expressed relative to the specific activity of glucose in the perfusion medium. For the determination of radioactivity, samples were added to Aquasol (New England Nuclear, Boston, MA) and counted in a Phillips PW 4510 liquid scintillation analyser.

3. Results and discussion

At 50 days of gestation the fetal guinea pig liver has a full complement of glycolytic and gluconeogenic enzyme activities (table 1) [3]. Pyruvate carboxylase is entirely mitochondrial, and its activity is relatively low. Phosphoenolpyruvate carboxykinase was largely if not exclusively mitochondrial [3], and its activity was relatively high.

Table 1
The activities of enzymes associated with glucose metabolism in the liver of the 48–51 days fetal quinea pigs

Enzyme	Activity (units/g wet wt)
Hexokinase (EC 2.7.1.1)	1.05 ± 0.22
Phosphofructokinase (EC 2.7.1.11)	4.13 ± 0.88
Triose phosphate isomerase (EC 5.3.1.1)	723 ± 245
Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)	34.6 ± 11.4
Pyruvate kinase (EC 2.7.1.40)	16.1 ± 5.3
Pyruvate carboxylase (EC 6.4.1.1)	0.21 ± 0.10
Phosphoenolpyruvate carboxykinase mitochondrial (EC 4.1.1.32)	1.84 ± 0.46
Fructose 1,6-bisphosphatase (EC 3.1.3.11)	9.6 ± 8.9
Glucose 6-phosphatase (EC 3.1.3.9)	3.7 ± 1.9
Glucose 6-phosphate dehydrogenase (EC 1.1.1.49)	2.1 ± 0.44

Results are for activity at 37°C and are means ± SD of 6–10 expt.

Here, a low glucose concentration (0.1 mM) was used in order to measure glucose uptake in the flow-through perfusion system. In the absence of other substrates glucose uptake was not increased at higher glucose concentrations, and in perfusions with 0.1 mM glucose, uptake remained relatively constant at ~40 nmol . min^{-1} . g^{-1}, after an initial 10–15 min. (fig.1). However the production of ^3H_2O from [2-^{3}H]-glucose was only ~2/3rds of its rate of uptake (table 2). The addition of a wide range of gluconeogenic substrates (e.g., lactate, pyruvate, alanine, serine, glycerol) did not result in glucose output. For instance, in perfusions with 10 mM dihydroxyacetone + 0.1 mM glucose, glucose uptake and ^3H_2O production from [2-^{3}H]glucose were depressed (fig.1, table 2). The glucose uptake and ^3H_2O production rates were comparable (fig.2), so there was no evidence for glucose 6-phosphatase activity despite the large increase in hexose phosphate concentrations brought about by dihydroxyacetone (table 3). The ^{14}C specific activity in glucose 6-phosphate was much lower in the presence of dihydroxyacetone, indicating that much of the glucose 6-phosphate was derived from the unlabelled substrate. The depression of glucose uptake can be explained by an inhibition of

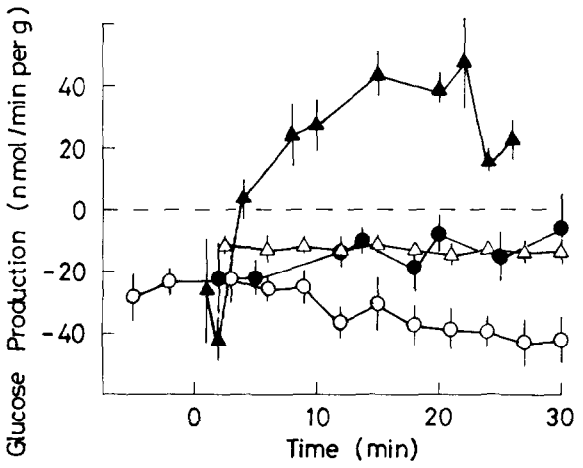

Fig.1. Glucose uptake/production in fetal liver perfusions. Livers of 49–51 day fetal guinea pigs were perfused with Krebs-bicarbonate buffer containing 0.1 mM glucose for 10 min, after which (time 0) the following additions were made to the perfusion medium: (A) 10 mM dihydroxyacetone; (•) 0.5 ng/ml glucagon; (▲/10 mM dihydroxyaceton + 0.5 ng/ml glucagon; (○) control (no addition). Glucose was assayed in perfusate samples to determine net uptake/production. Results are shown as mean ± SE for 3–7 obs.

hexokinase by the high glucose 6-phosphate concentration [10].

In perfusions with glucagon glucose uptake was depressed (fig.1), but ^3H_2O production was now higher than the glucose consumption rate (fig.2, table 2). This was coincident with an increase in hexose phosphate concentrations (table 3) and a fall in the $^3H/^{14}C$ specific activity ratio in glucose 6-phosphate, whilst ^{14}C specific activity was unchanged (table 2). These results suggest that glucose production had occurred, although there was still a net uptake of glucose. The glycogen concentration in 50 day fetal livers is <0.25 mg/g; during perfusion with glucagon it fell at a rate equivalent to ~20 nmol glucose . min^{-1} . g^{-1} over the first 20 min. If this glucose was released by the liver it would account for the apparent depression of glucose uptake. However, when livers were perfused with glucagon for 60 min the change in glycogen concentration between 40 and 60 min accounts for ~6 nmol glucose . min^{-1} . g^{-1} while the glucose uptake over this period was ~13 nmol . min^{-1} . g^{-1} and ^3H_2O production ~37 nmol . min^{-1} g^{-1}. Glycogen breakdown does not therefore account

Table 2
Glucose uptake and ^3H_2O production by, and glucose 6-phosphate specific activity in perfused livers of 49–51 day fetal guinea pigs

	Control	Glucagon (0.5 ng/ml)	Dihydoxy-acetone (10 mM)	Dihydoxyace-tone (10 mM) Glucagon (0.5 ng/ml)
Glucose uptake (nmol . min^{-1} . g^{-1})	38.6 ± 12.9	21.3 ± 17.8	15.2 ± 8.7	−29.1 ± 18.3
^3H_2O production from [2-^{3}H]glucose in glucose equiv. nmol . min^{-1} . g^{-1}	26.9 ± 7.6	35.6 ± 20.4	15.4 ± 5.9	8.5 ± 2.2
Rel. spec. act. in glucose 6-phosphate				
^{14}C from [U-^{14}C]glucose	0.23 ± 0.04	0.28 ± 0.11	0.12 ± 0.07	0.1 ± 0.001
^3H in 2-position (from [2-^{3}H]glucose)	0.076 ± 0.02	0.040 ± 0.02	0.015 ± 0.01	–
$^3H/^{14}C$	0.33	0.14	0.12	–

Livers were perfused with Krebs-bicarbonate buffer containing 0.1 mM glucose for 30 min. After 10 min [U-^{14}C,2-^{3}H]glucose was added to the perfusion medium, together with additions of glucagon and dihydoxyacetone as indicated above, and perfusion continued for a further 20 min. Glucose uptake and ^3H_2O production values refer to the last 10 min perfusion. The livers were then freeze-clamped and radioactive specific activities in glucose 6-phosphate were determined in $HClO_4$ extracts. These are expressed relative to the specific activity of glucose in the perfusion medium in each experiment, i.e., the ratio (dpm/nmol in glucose 6-phosphate)/(dpm/nmol in glucose). Results are means ± SD of 6–10 expt.

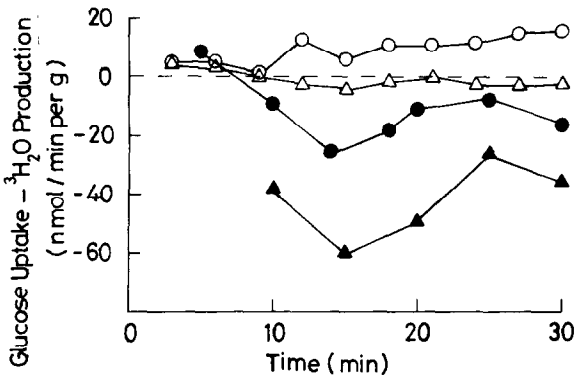

Fig.2. Glucose uptake–^3H_2O production in fetal liver perfusions. Livers of 49–51 day fetal guinea pigs were perfused as in fig.1. At time 0, [2-^{3}H]glucose (0.05 μCi/ml) was added to the perfusion medium together with: (△) 10 mM dihydroxyacetone; (●) 0.5 ng/ml glucagon; (▲) 10 mM dihydroxyacetone + 0.5 ng/ml glucagon; (○) control (label only added). Glucose uptake was as shown in fig.1; ^3H_2O production in glucose equivalents measured in perfusate samples was subtracted from the corresponding glucose uptake value.

for all the glucose produced by the glucagon-treated fetal liver.

In interpreting the difference between glucose uptake and ^3H_2O production, the extent of detritiation of hexose phosphate, which depends on the rate of equilibration at phosphoglucose isomerase relative to net flux [17] must be considered. In control perfusions detritiation is incomplete, as indicated by the $^3H/^{14}C$ ratio (table 2) and ^3H_2O production is less than the rate of glucose uptake, which in this situation probably represents the glucose phosphorylation rate. With dihydroxyacetone the lower $^3H/^{14}C$ ratio indicates nearly complete detritriation of hexose phosphates, and the ^3H_2O production is comparable to glucose uptake (table 2, fig.2). In this case both measurements are indicative of the glucose phosphorylation rate. With glucagon perfusions there is again a low $^3H/^{14}C$ ratio, but ^3H_2O production exceeds the net glucose uptake, as shown by the negative values in fig.2. The rate of glucose phosphorylation is unknown in this case, but clearly must be as great as, or greater than the ^3H_2O production rate. The explanation for net glucose uptake being lower than the phosphorylation rate is that glucose is produced via a functional activation of glucose 6-phosphatase. This conclusion is independent of the source of glucose 6-phosphate (i.e., whether it is glycogenolytic or gluconeogenic).

Glucose production was particularly apparent in perfusions with dihydroxyacetone and glucagon. There was a net glucose output of about 30 nmol $min^{-1} . g^{-1}$ (fig.1) and no significant change in liver glycogen concentration. Despite this, there was an uptake of glucose as measured by ^3H_2O production, giving a large negative difference between net glucose uptake and ^3H_2O production (fig.2). Glucose 6-phosphate concentration was further increased under these conditions, and the marked fall in its ^{14}C specific activity indicates that much of this can again be ascribed to an increased flux from unlabelled substrate. Thus in the presence of dihydroxyacetone there is clear evidence that glucagon not only causes a functional activation of glucose 6-phosphatase but also increases gluconeogenic flux.

In the adult rat liver glucagon causes changes consistent with control at phosphoenol pyruvate production [18], glucose 6-phosphate production [19] and inhibition of glycolytic flux [20]. While it is clear

Table 3
Metabolite concentrations in perfused fetal livers

	Control	Glucagon (0.5 ng/ml)	Dihydroxy-acetone (10 mM)	Dihydroxyacetone (10 mM) + glucagon (0.5 ng/ml)
ATP	1657 ± 236	1855 ± 221	1786 ± 213	1656 ± 328
ADP	324 ± 71	266 ± 123	248 ± 49	263 ± 102
Lactate	236 ± 69	294 ± 112	384 ± 32.9	489 ± 176
Pyruvate	34 ± 12.7	19.5 ± 13.2	43.5 ± 15.1	45 ± 19
Fructose 1,6-diphosphate	17.6 ± 4.9	19.1 ± 12	85.3 ± 28.2	71 ± 47
Glucose 6-phosphate	63 ± 21	138 ± 41.6	266 ± 5.3	412 ± 144
6-Phosphogluconate	3.7 ± 1.8	19.1 ± 12	85.3 ± 28.2	71 ± 4.7

Concentrations of metabolites were determined in $HClO_4$ extracts of freeze-clamped levers of 49–51 day fetal guinea pigs following 20 min perfusions under the conditions shown. The values are nmol/g wet wt, means ± SD of 6–10 expt.

that this hormone regulates pyruvate kinase [21] possible effects on phosphofructokinase/fructose 1,6-diphosphatase have not been proven [19,22]. If glucose 6-phosphatase activity is under short-term hormonal control the activation of this enzyme would exert a pull on gluconeogenic flux and would effectively reduce glycolytic flux. In fetal liver perfusions, 40–60 min after addition of glucagon, when there is a little glycogen breakdown, the glucose 6-phosphate concentration is still as high as after a 20 min perfusion, indicating that despite activation of glucose 6-phosphatase there was probably an increased gluconeogenic flux. If this was the case the change in glucose 6-phosphatase activity may have been caused by an increased availability of glucose 6-phosphate to the enzyme rather than an activation of the enzyme itself.

In the fetal guinea pig at ~50 days the plasma glucagon is 0.1–0.2 ng/ml. The insulin/glucagon ratio, however, is very high [9], and thus it is likely that no significant glucose synthesis occurs in vivo. The presence of glucose 6-phosphatase does mean that in response to stress the fetal liver could produce glucose, at least from glycogen [5,7,9]. It is likely that the fetuses of most species with a high insulin/glucagon ratio [9] behave in this way, and produce little glucose. The gluconeogenic pathway is probably required predominantly for glucose 6-phosphate production to maintain high rates of the pentose phosphate pathway at a time when hepatic glucose consumption is low and biosynthesis is high.

Acknowledgement

G.C.B. is a Medical Research Council Scholar.

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